(ORAL) CARDIOVASCULAR, PHARMACOLOGY and CHEMISTRY

#534

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

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638 THIRD AVENUE
NEW YORK, N. Y. 10017

COMMITTEE:
Dr. Reimann, Chm.
Dr. Andervont
Dr. Little

Application For Research Grant

Date: February 3, 1966

1. Name of Investigator:

Paul Goldhaber*, D.D.S. and Herbert I. Bader**, D.D.S.

2. Title:

* Associate Professor of Periodontology
** Research Fellow in Periodontology

3. Institution & Address:

Harvard School of Dental Medicine
188 Longwood Avenue, Boston, Massachusetts

4. Project or Subject:

See detailed sheets

The Production of Antibodies to Tobacco Smoke Condensate or Smoke in Animals and Humans

5. Detailed Plan of Procedure (Use additional pages if more space is required.)

See detailed sheets

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6. Budget Plan: See detailed budget sheet

a.	Salaries	
b.	Expendable Supplies	
c.	Other Expenses	
d.	Permanent Equipment	
e.	Overhead (15% of a, b, c)	
	Total	$ 24,414.00

7. Anticipated Duration of Work:

5 years

8. Facilities and Staff Available:

a) microscopic equipment with ultraviolet light source.
b) photographic equipment calibrated for fluorescent microscopy.
c) working immunological assay systems.
d) animal quarters.
e) all the facilities of the Dept. of Oral Histopathology & Periodontology at Harvard School of Dental Medicine.
f) Consultation and advice, whenever indicated by the personnel of the Harvard Medical Community.

9. Additional Requirements:

None presently

10. Additional Information (including relation of work to other projects and other sources of support):

Immunologic studies utilizing the fluorescent antibody technique as first developed by A. H. Coons[14] have been conducted in this laboratory for the past 3 years. These studies include both enzyme localization in tissues through the use of antibodies produced in rabbits against the enzymes in question and cytological localization of bacteria in human gingival tissues[16]. We feel there is direct applicability of the techniques developed in these studies to the problems posed in the present outline.

Currently, Dr. Bader's salary is derived from a U.S.P.H.S. Training Grant. His Fellowship will terminate June 30, 1966. The research supplies for the above mentioned research, equipment and technical assistance used are supported in part by the same training grant and a U.S.P.H.S. grant No. DE 01298 entitled "Further Studies of Mechanisms of Periodontal Disease" which is held by Dr. Paul Goldhaber.

Signature Paul C. Goldhaber
Director of Project

Business Officer of the Institution Henry C. Meadow, Exec. Secr.
2 Committee on Research and Development

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4. Project

The Production of Antibodies to Tobacco Smoke Condensate or Smoke in Animals and Humans

On the basis of clinical observation, morbidity and mortality tables of smokers and non-smokers and other selected groups, tobacco smoking has been associated with various pathological changes in the human organism. Further experimental data is necessary to clarify this association. One neglected aspect of the problem concerns the antigenicity of tobacco smoke and the pathological changes that may take place in the host.

It may be noted that where one can find reference to studies of the immunology of tobacco it is primarily tobacco extracts and not the combusted products (smoke) that are used as the antigenic agents.

Pipes[1] in 1945 first suggested that any demonstrable immunologic response to proteins extracted from tobacco leaves may be quite apart from hypersensitivity to tobacco smoke. Rosen in 1953[2] and 1954[3] pointed out the differences between tobacco leaf extracts and absorption of tobacco smoke by inhalation. It is difficult to imagine tobacco protein remaining stable and unaltered following combustion and thus acting as an antigenic stimulus in its natural form in the smoker. Some attempts at elucidating the immunological effects of tobacco smoke were made by Armen & Cohen in 1958[4] by studying electrocardiographic changes in rabbits sensitized to tobacco extracts and challenged by smoke. While normal and sensitized animals did react differently, the ECG changes did not appear to be immunologically related. While others[5,6] have commented on the antigenicity of tobacco smoke particularly as an aerosol there is a definite lack of information in the literature clarifying its properties as a challenging or shocking antigen in animals.

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As mentioned earlier the majority of reports in the literature are based on clinical studies and most of these relate to cutaneous hypersensitivity as elicited by tobacco extracts. Within the past few years an increasing amount of interest has been shown in the role of tobacco allergy in thromboangiitis obliterans (TAO) and coronary artery disease[7]. There is ample evidence from the above-mentioned studies and others that immediate wheal and flare[8] and delayed type reactions[9] can repeatedly be demonstrated in tobacco sensitized individuals using either tobacco leaf extracts or tobacco smoke condensate. While the above mentioned classical demonstrations of hypersensitivity can be duplicated in healthy individuals, patients with coronary artery disease and TAO have been shown to have a higher incidence of positive reactions to tobacco extracts[10,11], implicating in some fashion the possibility of tobacco allergy as being a factor in these cases. It is interesting to speculate on what role the immune system may play in the pathogenisis of the obstructive arteritis so typical of TAO (also known as Buerger's Disease).

The emphasis in the consideration of tobacco allergy as an accepted clinical entity and in the experimental work on the immunology of tobacco extracts[10,12] to this point in the discussion have all been based for the most part solely on the immunogenicity of the material (tobacco) without adequate investigation of other factors influencing host response. It is axiomatic that the ultimate immunologic response as judged by clinical or experimental criteria is to a great extent dependent upon the immunologic responsiveness of the host organism and the extent to which the response is mediated by existing disease, the nature of the antigen (e.g. - pharmacologic effects of nicotine) and any other parameters affecting the defense mechanism.

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In a review of the immunology of tobacco[13] there is evidence both for and against untoward influences on antibody formation by smoke and/or nicotine, the latter being

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non-antigenic although there may be some obscure haptenic activity. The pharmacologic effect of nicotine is a phenomenon quite apart from its antigenic properties. Tobacco smoke condensate if not antigenic in itself should be investigated for haptenic properties, particularly since it has been mentioned as having some properties as a shocking agent in eliciting anaphylaxis.

In summation, it appears the immunological aspect of smoking has been one of the most neglected phases of study in the relationship of tobacco utilization by the human organism. If we are to draw any valid conclusions or arrive at workable clinical concepts along this line these must be based upon fundamental immunological studies so as to provide a basis for expansion of our understanding of the nature of the tobacco smoke - host relationship.

If we accept the possibility that tobacco smoking may indeed be involved in the etiology of various diseases we still must explain why some smokers do not develop the disease in question whereas others do. It is conceivable that the difference may be in the immune response of the particular host to tobacco smoke. That is, the nature of the immune response may be the determining factor. Pathological changes may be the resultant of immune injury or may occur due to lack of immune protection. If the immune response is the key factor in precipitating the disease it may be possible to prevent or treat the disease in question by methods aimed at eliminating the responsible antigenic factor in tobacco smoke or by altering the host reaction. Furthermore, if our human studies indicate a correlation between the development of antibodies to certain components of tobacco smoke and the disease process, it may be possible to screen out those smokers who would fall into the category of "susceptible" individuals on the basis of their immunologic profile. Since non-smokers are known to develop all the diseases that have been associated with tobacco

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smoking we may postulate that the mechanism in those cases is similar although the antigen may be different.

With reference, therefore, to the paucity of experimental data on the immunogenicity of tobacco smoke, our long term goals are aimed at clarification of the immunological properties of tobacco smoke condensate in the human organism and to determine whether there is any correlation with various disease processes which seem to be associated with tobacco utilization. Specifically we intend to carry out antigenicity investigations in animals followed by cytological studies of antigen and antibody localization. At the same time we will study the nature of the smoke-host association in human subjects with emphasis on tissue changes as related to immune injury.

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5. Detailed Plans of Procedure

Experiments

1. Animal

Exp. 1.A Production of antibody in rabbits to tobacco smoke condensate.

Materials and Methods:

Ten female albino New Zealand rabbits about one year of age weighing between 2.5-3 kilos are divided into the following groups:

a) 6 animals to receive antigen and adjuvant

b) 2 animals to receive saline and adjuvant

c) 2 animals to receive saline

All animals are bled prior to immunization to insure non-reactivity between their serum globulins and the antigen (tobacco smoke condensate or TSC). The immunization schedule consists of TSC in 95% alcohol solution with a nitrogen content estimated by Lowry technique of 4 mg/cc administered over a series of 10 injections, to give a total of 40 mg. of nitrogen. The initial administration of 4 mg. of antigen nitrogen is combined with an equal volume of Freunds complete adjuvant and given with 1/2 dose into toe pad and 1/2 intraperitoneally. One week following the intial dose the animals are given 4 mg. of antigen nitrogen I.V. for 3 successive days, rested 4 days and then repeated twice for a total of 10 doses. One week following the tenth administration of antigen the animals are bled from the marginal ear vein and the serum portion of the blood separated. The presence of circulating antibody in the serum will be determined by gel diffusion and complement fixation studies.

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Exp. 1.B Production of antibody in guinea pigs to tobacco smoke condensate.

Materials and Methods:

Ten large (450 grams) guinea pigs are used in the following manner:

a) 6 animals are to receive antigen and adjuvant

b) 2 animals receive adjuvant and saline

c) 2 animals receive saline only

The choice of guinea pigs is based on the fact that they are well suited to studies in hypersensitivity particularly when dealing with a material whose immunogenicity is an unknown factor. While the animal is not a good producer of precipitating antibody it is excellent for screening purposes since a number of very sensitive tests for antibody formation can be performed in the same type of animal in which the hyperimmune state is being attempted. Such easily demonstrable antigen-antibody reactions as passive cutaneous anaphylaxis (PCA) are good indicators of immunogenicity. In addition the animals are small, easily handled and relatively inexpensive. Tests for production of antibody will include complement fixation and passive cutaneous anaphylaxis (PCA).

Exp. 2 Cytological localization of site of antigen (TSC) in tissues by fluorescent antibody technique.

Materials and Methods:

Five albino female New Zealand rabbits, 2.5-3K to be subjected to smoke by forced inhalation in a specially constructed smoke chamber[4], consisting simply of a wooden box in which the test animal is subjected to smoke from a cigar forced into the area by a stream of compressed air. There are suitable openings in the box to prevent suffocation of the animal.

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Source: https://www.industrydocuments.ucsf.edu/tobacco/

Animals thus exposed are then sacrificed and specific tissues are quick frozen in isopentane in liquid nitrogen. The tissues to be selected are:

1) Nasal mucosa
2) Respiratory tract including bronchi and alveoli
3) Lymphatics, particularly hilar lymph nodes
4) Liver
5) Spleen
6) Oral mucosa, including gingivae
7) Heart

Immunofluorescent study of sites of antigen localization are conducted by the indirect method of Coons[14].

All antiserum is adsorbed before use with dessicated tissue powder for non-specific or non-reactive material.

Tissue samples are sectioned on a cryostat at 2-4 microns and air dried on a slide. Fixation in 95% ethanol at 37°C for 15 minutes is followed by washing in pH 7.2 phosphate buffered saline.

Test slides are then covered with the adsorbed antiserum and incubated in a moist chamber for 1/2 hour, washed and dried.

Goat anti-rabbit globulin conjugated with fluorescein isothiocyanate is then layered over the specimen and incubated in similarfashion, washed, dried and coverslipped with 10% buffered glycerol as mounting medium.

Slides are examined under ultra-violet illumination provided by an Osram HBO-200 mercury vapor bulb with appropriate 1° and 2° filtration.

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Positive test for presence of antigen in the tissues is bright apple green fluorescence at site of union of antibody with the antigen.

Controls consist of:

1) Antiserum reacted with antigen (TSC) prior to use on slide to absorb all specific antibody.

2) Use of normal serum on tissue should result in negative test.

Exp. 3. Immunofluorescent localization of antibody to tobacco smoke condensate.

Materials and Methods:

Two female albino New Zealand rabbits are sensitized to tobacco smoke condensate as described in experiment #1.

Animals are sacrificed and tissues named in experiment #2 are obtained and frozen.

Tissues are sectioned at 2-4 microns on a cryostate and air dried on slides.

(TSC) Tobacco smoke condensate is conjugated by the method of Riggs et al[15].

The conjugated (TSC - fluorescein isothiocyanate) antigen is then layered over the tissue sections as in the direct fluorescent antibody technique of Coons[14].

General procedure is as outlined in experiment number 2.

Union of labeled TSC with specific antibody localized in the tissues is visualized as a bright apple green fluorescence.

Controls will consist of inhibition of antibodies by layering sections with unlabeled antigen prior to use of conjugate.

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Exp. 4. Sensitization of animals to tobacco smoke by inhalation.

Materials and Methods:

Five albino female New Zealand rabbits weighing 2.5-3 kilos are used as experimental animals.

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Each animal to be placed in smoke chamber daily for 15 minutes and subjected to smoke as described in earlier experiment.

At end of one week and once weekly for a period of time up to one year (if necessary) blood is to be drawn from marginal ear veins and serum tested for presence of anti-tobacco smoke antibody by complement fixation and gel diffusion.

Exp. 5. Investigation of haptenic properties of tobacco smoke condensate.

As mentioned in preceeding discussion if immunologic response is not elicited by the use of TSC alone it may be possible to conjugate the condensate with a protein, and then in the immunized animal evoke a response by challenging with the TSC.

Therefore a regimen of injections similar to that outlined in experiment #1 would be carried out with a TSC - protein combination using guinea pigs as the test animals. Haptenic activity could be demonstrated in the sensitized animal by evoking a passive cutaneous anaphylactic reaction with TSC.

II. Humans

Exp. 1. Demonstration of tobacco-smoke antibodies in serum of human subjects. Subjects to be screened for presence of circulating antibody by means of complement fixation and gel diffusion techniques. Both smokers and non-smokers to be included in screening. A minimum of 100 subjects to be tested, at least 1/2 being considered "heavy" smokers by generally accepted criteria.

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Exp. 2. Immunofluorescent localization of TSC antigen in oral tissues of smokers. Experiment to be followed as outlined in animal experiments (utilizing indirect test).

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Exp. 3. Immunofluorescent localization of antibody to tobacco smoke in sensitized human subjects.

All smokers found to have circulating antibody (in serum) to be further examined for presence of antibody in tissues with the direct fluorescent antibody test using TSC conjugated with fluorescein isothiocyanate.

All tissues in experiments 2 and 3 are to be oral tissues obtained by biopsy or at surgical phase of periodontal therapy, frozen and prepared as described previously.

Exp. 4. Patch testing of selected groups for cutaneous sensitivity to tobacco smoke.

Selected groups of smokers and non-smokers (as mentioned in Human experiment #1) to be exposed to smoke as a challenging stimulus by means of a cutaneous patch test. Observations to be recorded as either positive or negative hypersensitivity response.

All experiments are listed numerically and divided into two major groups e.g. animal and human. This is not meant to imply order of procedure or relative importance. It should be noted that attempts to immunize animals against tobacco smoke condensate can and should be performed concurrently with screening of human subjects for anti-tobacco smoke antibody. In addition, cytological localization of antibody (if any) is not dependent upon use of a prepared antiserum. As noted in experimental design an antigen source is the only requirement for this type of study.

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Source: https://www.industrydocuments.ucsf.edu/docs/nnwm0000

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BUDGET

Professional Personnel			
Paul Goldhaber, D.D.S., Associate Professor of Periodontology			0
Herbert I. Bader, D.D.S., Research Fellow in Periodontology		$9,000	
Soc. Sec. & Ret. Allow.	16%	1,440	10,440
Non-Professional Personnel			
Technical Assistance		5,000	
Secretarial Assistance		1,000	
		6,000	
Soc. Sec. & Ret. Allow.	9 1/4%	555	6,555
Consumable Supplies			
Animals and animal care		1,000	
Chemical reagents & glassware		600	
Histological supplies		750	
Photographic supplies		250	
Smoke chamber, tobacco smoke condensate & cigarettes		200	
Disposable syringes, needles, etc.		100	2,900
Travel			350
Publications			100
	Sub-Total		20,345
	Overhead 20%		4,069
	Total	$	24,414

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